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13. ABSTRACT ***(Maximum 200 Words)***

The androgen pathway is central to prostate tumorigenesis. An increased risk of higher stage, more aggressive prostate cancer is associated with a more active androgen receptor (AR). We are investigating and innovative transcription based mechanism that represses AR activity *in vitro*. Our hypothesis is that the tumor suppressor gene, WT1, may play a role in prostate tumorigenesis mediated by repression of AR gene expression. To validate our AR promoter data we demonstrated that AR target gene down-regulation by WT1 is dependent on an intact DNA binding domain, is mediated by AR and is hormone dependent. Additionally we confirmed our RNA studies showing that WT1 protein expression patterns are inversely related to AR expression. Androgen responsive cell lines express AR but fail to express WT1, while androgen independent lines express WT1 and lack AR, suggesting a correlation with late-state androgen independence. Recently we established stably transfected tumor cell lines and are now determining their growth characteristics with the intent of using them to establish a mouse model of prostate cancer progression. With the correlation of WT1 expression with higher grade disease and the potential to demonstrate WT1 repression of AR expression in mice, we will establish the role of WT1 in the development of androgen independence.

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Introduction

The androgen pathway is central to prostate tumorigenesis. An increased risk of higher stage, more aggressive prostate cancer is associated with the inheritance of a more active androgen receptor (AR) (1-3). We are investigating an innovative transcription based mechanism that represses AR activity *in vitro*. The tumor suppressor gene, WT1, transcriptionally represses many growth control genes including AR. Our hypothesis is that WT1 may play a role in prostate tumorigenesis mediated by repression of AR gene expression. We have previously demonstrated that WT1 repression of the AR gene promoter construct (4) is mediated by DNA binding. To validate these exogenous AR promoter data we demonstrated that WT1 represseses the endogenous AR promoter in androgen responsive cells (5). Now we have demonstrated that this AR target gene down-regulation by WT1 is dependent on an intact DNA binding domain, is mediated by AR and is hormone dependent. Additionally using Western blot analysis we confirmed the inverse relation between expression of WT1 and AR in prostate cancer cell lines, previously demonstrated by RT-PCR analysis. Androgen responsive cell lines express AR but fail to express WT1, while androgen unresponsive lines express WT1 and lack AR, suggesting a correlation with late-stage androgen independence. Thus, our hypothesis that WT1 transcriptionally represses AR gene expression in WT1-producing prostate tumor cells is being tested using both in vitro and in vivo approaches which assess both the significance of WT1 expression in prostate tumors and the mechanism of AR repression. With our recent establishment of stably transfected LNCaP lines we will now be able to establish a mouse model of prostate cancer progression. If the correlation of WT1 expression with higher grade disease is upheld in on-going studies and if WT1 represses AR expression in vivo, then we will have established a model of WT1-mediated progression of prostate tumors to androgen independence.

BODY OF WORK

We have demonstrated an inverse correlation between WT1 and AR expression in several prostate tumor cell lines, suggesting that WT1 may play a role in prostate tumorigenesis. This hypothesis is supported by previous observations by others that in prostate tumor biopsies a significantly higher percentage of tumor cells (30%) expressed WT1 protein than did adjacent normal cells (12%) and normal prostate biopsies showed no significant WT1 expression (6). Our recent work (discussed below) has confirmed and extended these previous findings.

PREVIOUS RESULTS:

1. WT1 and AR expression are inversely correlated

The expression of AR during fetal and postnatal development in androgen target tissues is inversely correlated to WT1 expression. Using RT-PCR we previously confirmed this inverse relationship of WT1 and AR expression in the prostate, an androgen responsive tissue. In collaboration with Dr. Nora Navone, we determined that WT1 is not expressed in LNCaP and MDAPCa2b, two androgen-responsive prostate tumor cell lines expressing AR mRNA. Conversely, WT1 is expressed in DU145 and PC3; two androgen unresponsive, highly tumorigenic prostate tumor cell lines that lack AR expression. If WT1 also represses the AR gene promoter in vivo, then inhibition of AR-induced transactivation of AR target genes may suppress tumor cell growth in androgen-responsive prostatic tumor cells. This work has been extended by examining protein expression levels in the prostate tumor cell lines and in the tumor progression cell lines derived from both LNCaP and PC3 prostate cancer cell lines.

2. WT1 repression of the AR promoter is mediated by DNA binding

In previous work we have demonstrated 3-10-fold repression of the AR promoter by overexpression of WT1(-KTS) isoforms in transient transfection assays in HeLa, T47D (Breast cancer), SaOS (osteosarcoma), 293 (kidney) and TM4 (Sertoli) cells. The specificity of this repression was confirmed by demonstrating the inability of a zinc-finger mutant WT1 expression construct to repress the AR promoter construct in HeLa cells. Thus, WT1 repression of the AR promoter requires a functionally intact DNA binding domain. This repression was demonstrated by electrophoretic mobility shift assay (EMSA) to be mediated by binding of the WT1 protein to at least two regions containing 6 potential WT1 binding sites in the AR promoter. This work has been extended by searching for other possible mechanisms of interaction between WT1 and AR, such as protein-protein interactions. We co-transfected both AR and WT1 expression constructs into HeLa and Cos cells and examined the effect on downstream targets as well as directly assessing physical interactions by co-labeling studies.

3. WT1 repression of the AR pathway

For a better understanding of the role played by WT1 in regulating the expression of AR and AR-target genes, we examined the effect of overexpression of WT1 in 293 kidney and TM4 Sertoli cells expressing AR. In these assays the ability of WT1 to directly repress the endogenous AR promoter resulted in an indirect repression of an AR-target gene construct containing four copies of the ARE binding site (GGTACAnnnTGTTCT), the E1B TATA box and the luciferase gene. WT1 over-expression in 293 and TM4 cells down-regulates AR and indirectly results in a dose-dependent reduction in ARE-activated luciferase activity. These experiments have been extended to prostate tumor cells, LNCaP and MDAPCa2b. This has allowed us to confirm this indirect mechanism by dual labeling immunofluorescence assays of WT1 and AR expression in LNCaP cells transiently transfected with WT1. We have reproduced these experiments in a subline of LNCaP cells (obtained from our collaborator, Dr. G. Jenster) stably transfected with WT1-FLAG expression constructs (obtained from another collaborator, Dr. C. Roberts).). The stably transfected lines allow quantitative western blot analysis using both WT1 and FLAG Ab.

These previous results demonstrated that: 1) WT1 and AR expression are inversely correlated in androgen target tissues, 2) WT1 protein binds at least two of the WT1 binding sites in the AR promoter in vitro, 3) WT1 directly binds and represses exogenously added AR promoter constructs in kidney and gonadal cells, and 4) WT1 repression of the endogenous AR promoter in kidney and gonadal cells interferes with the androgen signal transduction pathway causing down-regulation of AR target gene transcription.

TECHNICAL OBJECTIVE I. To determine the prognostic significance of WT1 expression in prostate tumors.

Is WT1 protein expressed in prostate epithelial cells ?

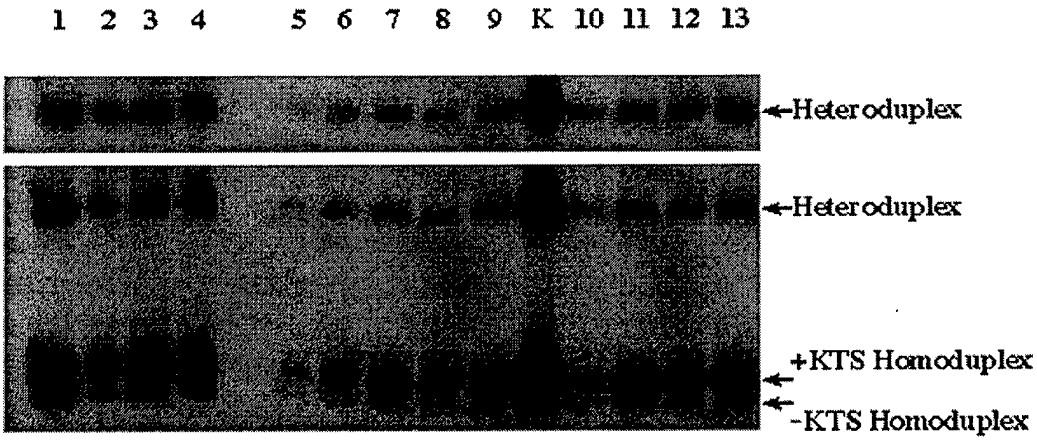
Stage and grade of prostate tumors were determined by medical record examination and correlated with WT1 expression. Previous analysis demonstrated that WT1 expression correlated with high grade and stage tumors. Gleason 5 grade tumors showed no WT1 expression, while some Gleason 9 grade sections displayed focal epithelial cytoplasmic staining, sometimes accompanied by nuclear staining but often not. Normal prostate tissues and low grade PIN samples lacked WT1 expression (data not shown). However with increased analyses of patient samples we have now observed rare

staining in both Gleason Grade 5 tumors and in non-prostate epithelial cells (Figure 1 and Figure 2). Using anti-WT1 Ab (C19, Santa Cruz) both dark brown staining nucleii and light brown staining cytoplasm are visible in prostate tumor epithelial cells. Primarily we observed focal cytoplasmic staining of ephithelial cells of high grade prostate tumor sections (Gleason 9) and rarely staining of low grade tumor sections (Gleason 5) with both cytoplasmic and nuclear staining (**FIGURE 1,** in appendix). Right panels shows specificity of staining, as all staining is blocked by treating the Ab with WT1 blocking peptide prior to incubation with tissues.

WT1 expression in the adult kidney podocytes is nuclear however, in adjacent tubular epithelial cells expression is cytoplasmic. Similarly both cytoplasmic and nuclear staining has been observed with the polyclonal Ab. Some areas showing WT1 staining include seminal vessicles, muscle fibers, squamous epithelium, and polymophonuclear cells. Additionally we have observed stromal cells with cytoplasmic staining.

Is WT1 protein found in both nucleus and cytoplasm? In an effort to validate the previously observed cytoplasmic staining of prostate tissue sections, we examined adult mouse kidneys. We observed nuclear staining restricted to podocytes, but also clear evidence of cytoplasmic staining in the adjacent tubular epithelial cells (**FIGURE 2A,** in appendix). Similarly both cytoplasmic and nuclear staining has been observed with the monoclonal Ab (data not shown). Additionally, western blot analysis of nuclear extracts and cytoplasmic remnants (supernatant removed from nuclear pellets) showed the presence of WT1 protein in both the nuclear and cytoplasmic fraction of androgen insensitive cell lines: DU145, LNCaP-LN3, PC3, and PC3 sublines (data not shown). We confirmed the cytoplasmic location of WT1 protein using the same polyclonal anti-WT1 Ab (C19, Santa Cruz) in immunohistochemical analyses of prostate epithelial cell lines grown in 8-well chamber slides. We observed dark brown staining nucleii and light brown staining cytoplasm in the androgen insensitive cell lines (Data not shown). Specificity of staining was confirmed by treating Ab with WT1 blocking peptide prior to incubation of the polyclonal Ab with cell lines. While WT1 expression is not limited to the nucleus, it's function (if any) in the cytoplasm is unknown.

Finally WT1 staining is not limited to prostatic epithelium, as we have now observed stromal cells with cytoplasmic staining. Additionally other tissues in which we have observed WT1 staining include seminal vessicles, muscle fibers, squamous epithelium, and polymophonuclear cells (**FIGURE 2B-D,** in appendix).

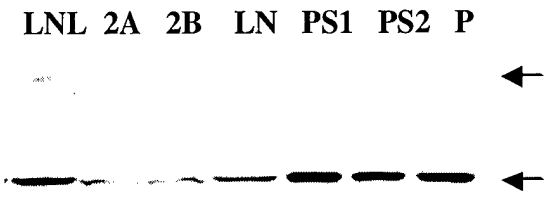


Is expressed WT1 protein normal or mutated?

The observed overexpression of WT1 in prostate tumor tissues does not preclude the possibility of the absence of

normal WT1 mediated tumor suppression. To determine whether the prostatic expression is normal WT1 or mutant, we need to develop a rapid screening method for identification of naturally occurring zinc finger mutations of the WT1 gene. Thus we developed the methodology and established conditions necessary for heteroduplex analysis of WT1 exons 7-10 in tumor tissue RNA. Initially we have analysed leukemic buffy coat samples that overexpress WT1, as determined by RT-PCR analysis. This overexpression in acute myelogenous leukemia is in contrast to normal lymphocytes which fail to express significant levels of WT1. To date, we have observed no evidence of mutation in the zinc finger region. If unexpected isoform ratios are observed in mRNA isolated from cultured prostate tumor cells, then we will obtain frozen prostate tumor tissue for RNA analysis.

Does WT1 protein correlate with androgen sensitivity prostate cancer cells?



Nuclear extracts containing 30 ug of protein were separated by electrophoresis through a 12% SDS-polyacrylamide gel and analyzed by Western blotting using WT1 Ab (c19, Santa Cruz). Immunoreactive proteins were visualized by ECL-Plus luminescence and autoradiography. High salt extracts were prepared from the following cell lines: LN, LNCaP; 2A, MDAPCa2a; 2B, MDAPCa2b; PS1, primary cultures of mouse stromal cells ; PS2; P3; These protein assays confirmed our RNA expression studies showing that WT1 expression patterns are inversely related to AR expression. Androgen responsive normal mouse prostate stromal lines and tumor cell lines express AR but fail to express WT1, while androgen independent lines express WT1 and lack AR. The androgen independent LNCaP-C42 (not shown) and LN-LN3 variants express WT1 (LNL), while the androgen dependent LNCaP (LN), MDAPCa2a and MDAPCa2b cells lack WT1, suggesting a correlation of WT1 expression with late-stage androgen independence.

To further explore the relationship of androgen responsiveness to WT1 expression, we examined LNCaP and PC3 sublines derived by serial subpassage of these cells in either lymph nodes (L) or prostates (P) of nude mice. Cell extracts containing 30 ug of protein were analyzed by Western blotting and visualized by ECL-Plus luminescence and autoradiography. LN, LNCaP; LNL, LNCaP-LN3; LNP, LNCaP-Pro5; KAL, marker; P, PC3; : PCL, PC3-LN; PCP, PC3-Pro. The position of the 45-47 kd WT1 proteins is marked with an arrow. Note that WT1 expression is higher in the PC3 and LNCaP sublines than in PC3 and LNCaP.

Interestingly LNCaP-Pro5, previously reported to be androgen sensitive (9), does express some WT1. However, using a dual labelling assay (see below) we have developed for WT1 and AR co-expression studies using TRITC-anti-rabbit and FITC anti-mouse antibodies we observed an inverse relation of WT1 to AR in these cells as well (Fig 3, Appendix). LNCaP cells express AR (FITC staining in left panel) and 5nM R1881 strongly induces AR immunofluorescence in LNCaP (FITC staining in right panel) But no WT1 staining is observed. In contrast LNCaP-Pro5 cells express AR only weakly (FITC staining in left panel) but do express WT1 in some cells (TRITC staining in left

panel). Interestingly, in our hands, this androgen sensitive line is not highly responsive to R1881 induction. While 5nM R1881 strongly induces AR immunofluorescence in LNCaP it only weakly induces AR in LNCaP-Pro5. (FITC staining in right panel). However, the AR positive LNCaP-Pro5 cells lack WT1 and the WT1 positive LNCaP-Pro5 cells lack AR expression. Thus, AR and WT1 are not co-expressed in the same cells as shown by the lack of yellow staining with the dual staining procedure.

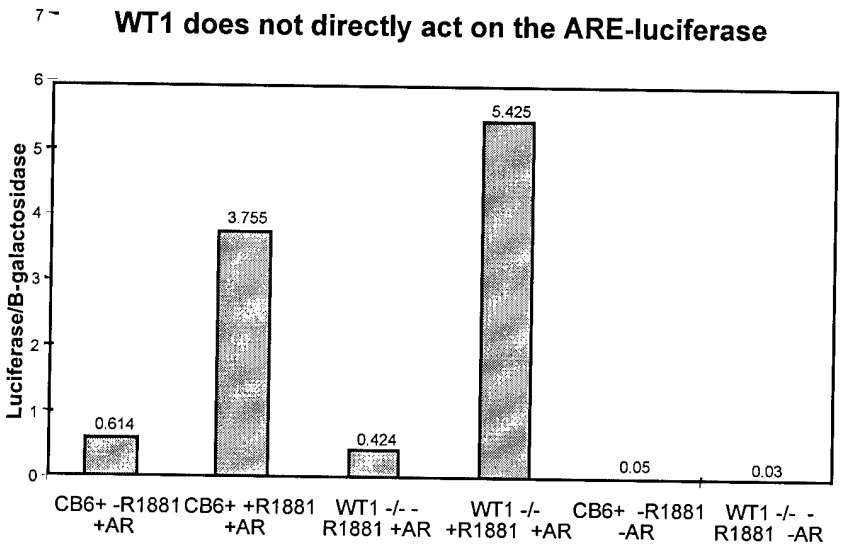
We confirmed these results by western blot analysis of LNCaP, LNCaP-LN3, and LNCaP-Pro5 progression lines using monoclonal antibody obtained from a different source (data not shown). Initially we probed the filter with a mouse mAb (DAKO) which detects an amino terminus peptide of WT1. Then we stripped the western blot and reprobed with the previously described polyclonal Ab which detects a carboxy terminus peptide. Finally we stripped and reprobed the western blot with the polyclonal Ab which had been preincubated with blocking peptide (the immunogen). The mAb and the pAb gave identical results and the pAb was clompletely blocked by preincubation with the peptide. Thus, unlike LNCaP cells, the more aggressive LNCaP sublines express WT1, although on a single cell level (FITC/TRITC dual labelling), WT1 expression is inversely related to AR.

Overall, our results are in agreement with those showing primary cultures of normal epithelial cell strains lack significant WT1 expression (7). However, our results do not support the findings of others that some normal prostate stromal cell strains do express WT1, as detected by RT-PCR and RNAse PA (7). In our primary cultures of mouse stromal cells, we saw no evidence of WT1 expression but did observe androgen response and strong AR expression. Similarly, the reported lack of WT1 expression in epithelial tumor cell strains, but presence in stromal tumor cell strains (7), is surprising as high WT1 expression levels seen in nephroblastomas are restricted to epithelial predominant tumors and are reduced in stromal predominant tumors (8). We have now observed WT1 expression rarely in stromal tissues of prostate tumor sections but not in our cultred cell lines. However we do observed WT1 in several epithelial prostate tumor cell lines, particularly the more aggressive, androgen unresponsive lines.

TECHNICAL OBJECTIVE II. To determine whether WT1 represses the androgen signal transduction pathway in prostate tumor cell lines.

Several explanations for the inverse relationship of WT1 and AR expression are possible. One is that WT1 binds AR and thus blocks binding of AR to its cognate binding site, the ARE. A second possible explanation is that WT1 may directly the ARE site and block AR from binding to the ARE. We examined both of these potential mechanisms, first we searched for evidence of protein-protein interactions between WT1 and AR. Using a dual labelling assay we have developed for WT1 and AR co-expression studies we incubated the cotransfected monolayers (grown on glass slides) with monoclonal anti- FLAG Ab and polyclonal anti-AR Ab followed by incubation with TRITC-anti-mouse and FITC anti-rabbit antibodies. We detected TRITC-labeled (**Fig 4, WT1-Ab,** central panels, Appendix)and FITC-labeled cells (**Fig 4, AR-Ab,** left panels, Appendix) and some dually-labeled cells (**Fig 4, Overlaid images,** right panels, Appendix). One possible mechanism of interaction between two transcription factors could result from their binding at adjacent sites on DNA. To determine whether colocalization of AR and WT1 might require the AR DNA binding domain we repeated these studies using a mutant AR expression construct deleted for the DNA binding domain but retaining the hormone/ligand binding domain (construct kindly provided by our collaborator Dr. Guido Jenster). In

the presence of hormone the mutant AR was still localized to the nucleus and was colocalized with WT1 (as detected by the yellow color in the top far right panel, **Fig 4**).



We then ruled out the possibility that WT1 was directly binding the ARE by transfecting cos cells with the ARE-luciferase target and WT1(-/-) without AR. As expected WT1 alone failed to activate the ARE-luciferase target (right two bars), while cotransfection of the empty vector CB8+ and the AR in the presence of hormone R1881 (2nd bar from left) activated the ARE-luciferase target 3x. Likewise, in the presence of hormone (R1881) WT1 does not block AR protein from activating the ARE. We demonstrated that the AR expression construct is activated 5x by the exogenously added AR expression construct even in the presence of exogenously added WT1(-/-) expression construct (4th bar from left). Thus, WT1 repression of AR downstream targets cannot be mediated by protein-protein interactions in these co-transfection assays.

Our dual labelling experiments of LNCaP, androgen responsive prostate tumor cells, has allowed us to exclude direct physical interaction of WT1 and AR and supports our earlier data suggesting an indirect mechanism of endogenous AR repression by WT1. Using dual labeling immunofluorescence assays of WT1 and AR expression in LNCaP cells transiently transfected with WT1 we also demonstrated inverse expression. . Transfection efficiency in LNCaP cells is so low as to preclude bulk cell analysis of transiently transfected monolayers. However, we have reproduced these experiments in stably transfected LNCaP cells allowing quantitative western blot analysis of WT1 and AR expression in the transfected LNCaP cells. We stably transfected a subline of LNCaP cells (obtained from our collaborator, Dr. G. Jenster) with WT1-FLAG expression constructs (obtained from our collaborator, Dr. C. Roberts). These include the normal (-)KTS isoforms and the DDS zinc finger mutant (-)KTS isoforms which fail to bind DNA. The stably transfected lines grown in the presence or absence of hormone allow quantitative western blot analysis using WT1, AR and FLAG Ab. We observed that WT1 protein expression is high in the WT1 (-/-) and WT1-DDS (-/-) mutant transfected lines, but is absent in the pCDNA3.1 vector transfected and parental LNCaP lines. Significantly we see AR induction by R1881 hormone treatment greatly increases AR levels in parental lines but no significant increase is seen in either DDS or wild-type WT1 transfected lines (data not shown).

Additionally we have established non-inducible WT1-FLAG tagged stably transfected MDAPCa2b cell line (performed in collaboration with Dr. Navone). These cells are androgen responsive, PSA expressing cells that like LNCaP contain mutant AR with slightly altered hormone affinity. Because we observed poor growth of these lines we have not yet obtained protein for western blot analysis and are also continuing our efforts in establishing regulatable WT1 expressing lines (which may grow better in the absence of WT1).

TECHNICAL OBJECTIVE III. Establish a mouse model for prostate cancer progression.

We have established stably transfected lines over-expressing the (-/-) isoform of wild-type WT1 or theDDS zinc finger mutant and have determined their growth characteristics and are using them to establish a mouse model of prostate cancer progression. While no obvious morphological differences have been observed between WT1, DDS and vector transfected lines, we do observe a slight growth suppression of the WT1 transfected lines (significantly slower than parental lines, only). Thus, we anticipate a slight increase in the normally long tumor growth latency period in vivo (12). To date we have injected several LNCaP cell lines stably transfected with WT1-FLAG expression constructs (obtained from our collaborator, Dr. C. Roberts). We expect tumor formation within 4-6 more weeks, as preliminary results indicated a two-month lag time for tumor development of parental LNCaP lines. Currently we are waiting for tumor development in nude mice inculated subcutaneously with matrigel suspended cells (1:1). We have examined LNCaP mock transfected, vector transfected, WT1 (-/-) and DDS (-/-) transfected cells. If we observe significant tumor growth enhancement, then we will repeat these experiments using cells without matrigel.

KEY RESEARCH ACCOMPLISHMENTS

1. **WT1 protein is strongly expressed in androgen insensitive prostate cancer cell lines , but not in androgen responsive lines prostate cancer cell lines.**

2. **WT1 protein is focally expressed in both nucleus and cytoplasm of high grade prostate tumor sections and in rarely in moderate grade tumors.**

3. **LNCaP cell lines stably transfected with FLAG WT1 (-/-) and FLAG-DDS WT1 (-/-) have been established and characterized.**

4. **LNCaP cell lines stably transfected with FLAG WT1 (-/-) and FLAG-DDS WT1 (-/-) have been injected subcutaneously into nude mice.**

Published Abstracts:

Annamaria Zaia, Ryuji Shimamura, Grady Saunders, **Gail Fraizer**,
Regulation of the Androgen Receptor by a Tumor suppressor gene, WT1. AUA 1999 Annual meeting, Dallas, Texas, 1999

Gail Fraizer, Miguel Diaz, Annamaria Zaia, Ryuji Shimamura, Grady Saunders
The Androgen Receptor is repressed by WT1, a Tumor suppressor gene. March 19-24, 2000. Keystone Symposia: Advances in Human Breast and Prostate Cancer, Nevada, 2000.

CONCLUSIONS

If WT1 transcriptionally represses AR gene expression in WT1-producing prostate tumor cells, two possible predictions can be made. 1) Overexpression of a tumor suppressor and subsequent repression of the AR pathway could lead to tumor suppression. Or 2) If suppression of AR confers a growth advantage selecting AI tumor cells, then we would predict an outgrowth of aggressive hormone refractory tumor cells causing tumor progression. In the latter case, growth advantages conferred by the repression of the AR by WT1 lead to androgen independence. The hypothesis that WT1 can

mediate androgen independent tumor progression is being tested using both in vitro and in vivo approaches which will assess both the significance of WT1 expression in prostate tumors and the mechanism of AR repression. The first two specific aims were performed concurrently and the stably transfected LNCaP and MDAPCa2b lines have been evaluated for their growth characteristics with the intent of using them to establish a mouse model of prostate cancer progression. We are currently determinng whether any tumors established by stably transfected LNCaP lines will progress to androgen independence. This will test our hypothesis that WT1 represses AR expression in vivo. Together these results will support our hypothesis that WT1 plays a role in the progression of prostate tumors to androgen independence.

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Figure 1

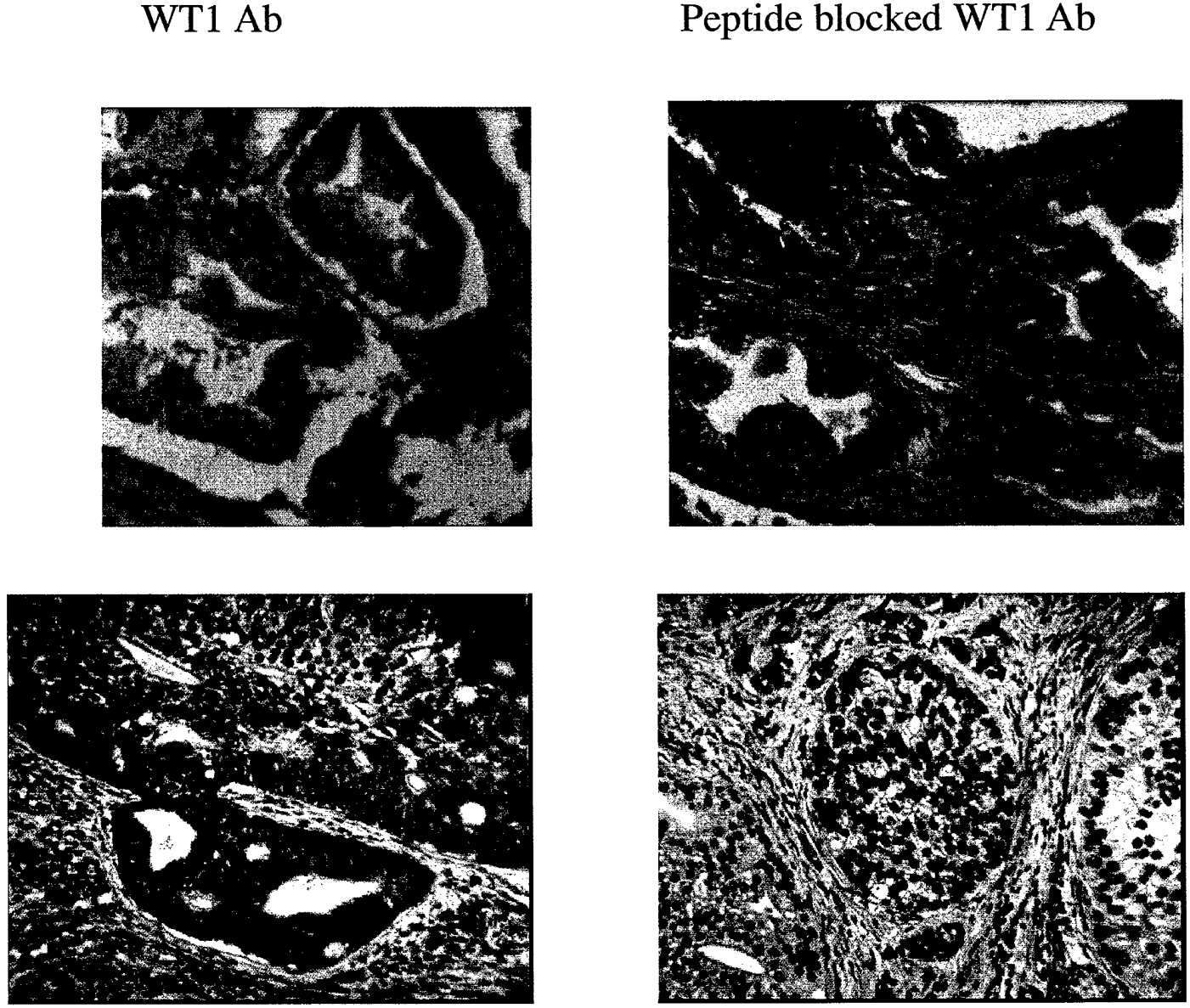
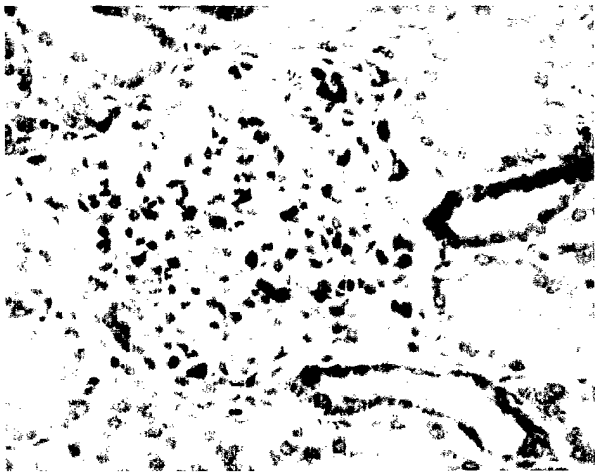


Figure 2

WT1 expression not limited to prostatic epithelium

A.



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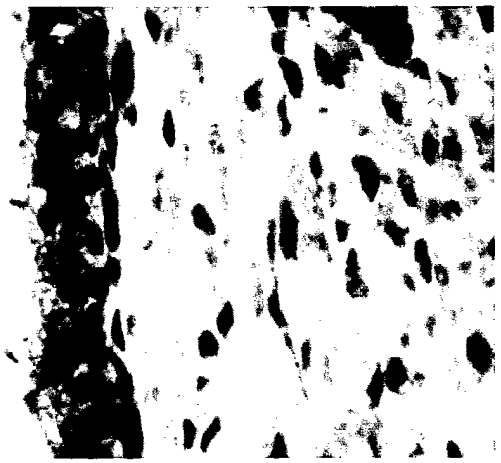

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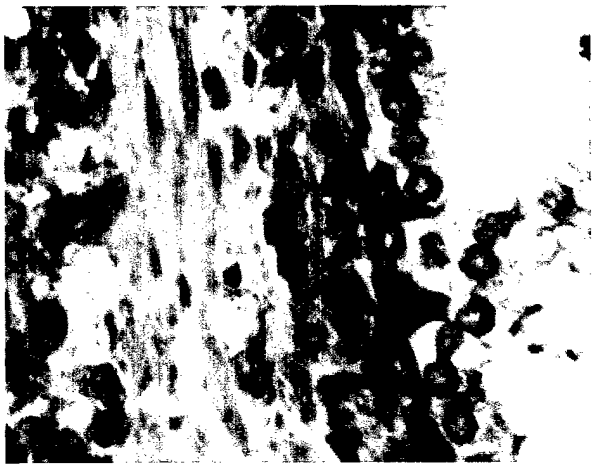

D.

Fig. 3

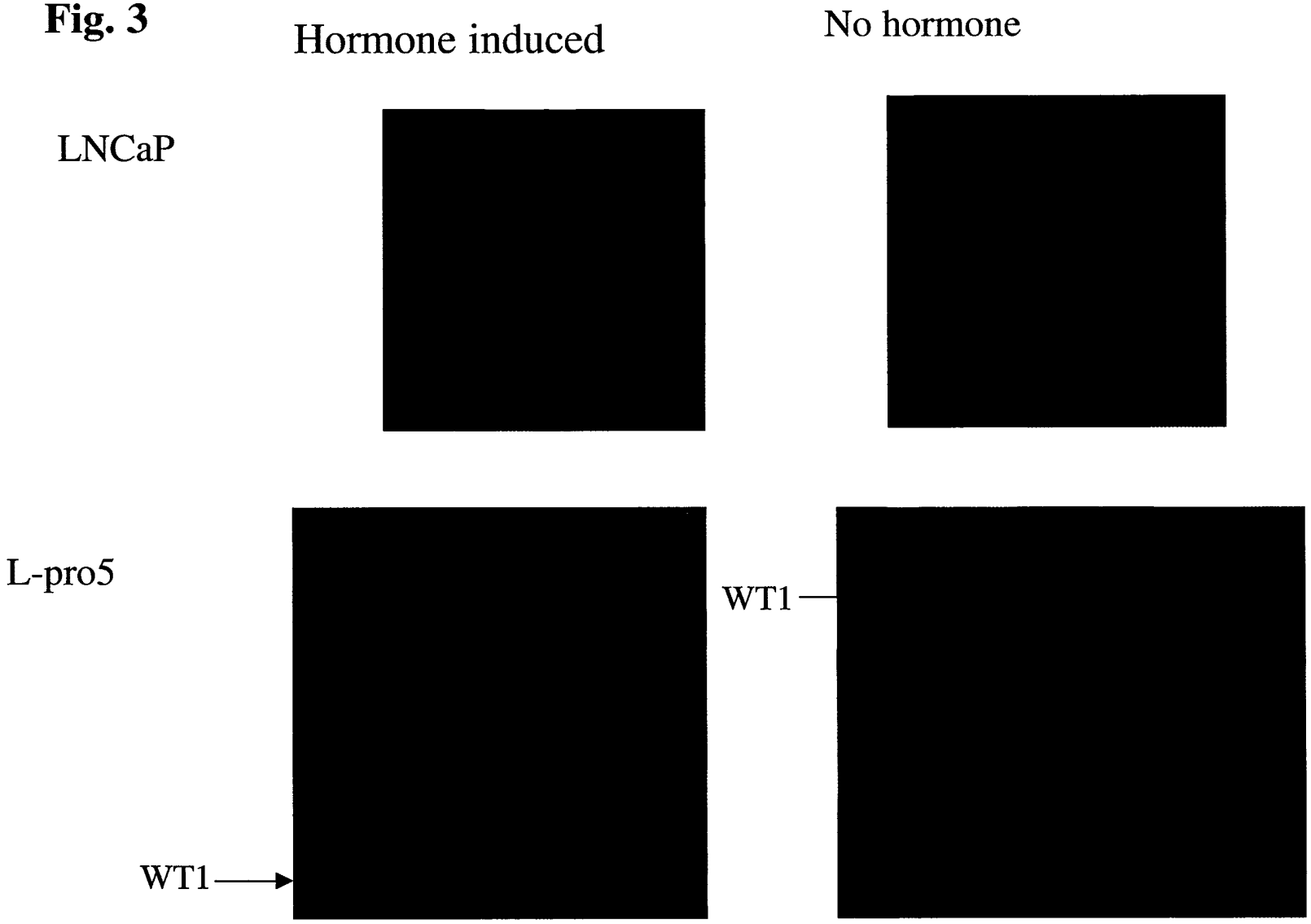

Figure 4

Co-localization of WT1 and AR in co-transfected COS cells does not require the DNA binding domain